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Note

Chemical constituents from the roots of *Cynanchum paniculatum* and their cytotoxic activity

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ABSTRACT

Two new steroidal saponins, cynanside A (**1**) and B (**2**) were isolated together with three known compounds (**3**–**5**) from the roots of *Cynanchum paniculatum* (Bunge) Kitag. The structures of **1** and **2** were elucidated on the basis of spectroscopic analyses, including extensive 2D NMR and acid hydrolysis. We evaluated the cytotoxicity of isolates (**1**–**5**) against A549, SK-OV-3, SK-MEL-2, and HCT-15 cell lines in vitro using the SRB bioassay. Compounds **1** and **2** showed selective cytotoxicity against SK-MEL-2 cells with IC_{50} values of 26.55 and 17.36 μM, respectively.

Steroidal saponins have been isolated from various plant sources. They possess a wide range of pharmacological activities including cytotoxicity.[1–5] Among them, C21 steroidal saponins from the genus *Cynanchum* exhibit antitumor and cytotoxic activities.[6–9] As a part of our ongoing search for cytotoxic constituents from Korean medicinal sources, MeOH extract of the roots of *Cynanchum paniculatum* (Bunge) Kitag (Asclepiadaceae) was selected for phytochemical investigation because the *n*-hexane and $CHCl_3$-soluble fractions showed cytotoxic activity using a SRB bioassay in our screening procedures. Our previous phytochemical investigation on MeOH extract of this source resulted in the isolation of seven C21 steroidal saponins.[10] In our continuing study on this source, we further isolated two new C21 steroidal saponins (**1** and **2**) from the active $CHCl_3$-soluble fraction and three known compounds (**3**–**5**) from the *n*-hexane-soluble fraction. Their structures were elucidated by 1D and 2D NMR, HR-FAB-MS, acid hydrolysis, and GC/MS. We also evaluated the cytotoxicities of isolates **1**–**5** (see Fig. 1).

Cynanside A (**1**) was obtained as an amorphous gum. The molecular formula was determined as $C_{42}H_{64}O_{15}$ from the molecular ion peak $[M+Na]^+$ at *m*/*z* 831.4144 (calcd for $C_{42}H_{64}NaO_{15}$, 831.4143) in positive ion HR-FAB-MS. The ^{13}C NMR data (Table 1) in combination with analysis of the DEPT and HMQC spectra revealed 42 carbon signals due to eight methyls, nine methylenes, 20 methines, and five quaternary carbons, of which 22 were assigned to the aglycone part including one carbonyl carbon (δ_C 179.3) and four olefinic carbons (δ_C 142.2, 140.0, 131.1, and 120.1). The remaining 20 carbons were ascribed to three sugars and two methoxy groups. The NMR spectral data including ^1H NMR, DEPT, ^1H–^1H COSY, HMQC, and HMBC spectra showed the presence of two oxygenated methines [δ_H 5.73 (1H, ddd, *J* = 8.0, 7.5, 5.0 Hz, H-16), 3.75 (1H, m, H-3)], one acetal methine [δ_H 5.59 (1H, s, H-18)], two olefinic methines [δ_H 5.48 (1H, dd, *J* = 11.0, 5.0 Hz, H-12), 5.37 (1H, m, H-6)], one oxygenated methylene [δ_H 4.42 (1H, dd, *J* = 10.0, 7.5 Hz, H-15a), 4.12 (1H, dd, *J* = 10.0, 5.0 Hz, H-15b)], one methoxy group [δ_H 3.49 (3H, s, H-18-OCH_3)], and two methyl groups [δ_H 1.71 (3H, s, H-21), 0.95 (3H, s, H-19)] of the aglycone part, and three anomeric protons [δ_H 5.55 (1H, dd, *J* = 10.0, 2.0 Hz, H-1″), 5.25 (1H, d, *J* = 3.0 Hz, H-1‴), 4.81 (1H, dd, *J* = 10.0, 2.0 Hz, H-1′)], two methoxy groups [δ_H 3.57 (3H, s, H-3′-OCH_3), 3.33 (3H, s, H-3‴-OCH_3)], and three methyl groups [δ_H 1.51 (3H, d, *J* = 6.5 Hz, H-6‴), 1.45 (3H, d, *J* = 6.0 Hz, H-6″), 1.45 (3H, d, *J* = 6.0 Hz, H-6′)] of the sugar moiety. These spectroscopic data of the aglycone part were similar to those of stauntogenin 3-*O*-α-oleandropyranosyl-(1→4)-β-digitoxopyranosyl-(1→4)-β-oleandropyranoside[10] except for the replacement of carbonyl carbon (δ_C 167.5) with acetal carbon (δ_C 104.2), and the presence of an additional methoxy carbon (δ_C 55.0). This was confirmed by HMBC correlations of H-18-OCH_3/C-18, H-18/C-12, and C-20 (Fig. 2). The sugar moiety was assumed to be α-L-oleandropyranosyl-(1→4)-β-D-digitoxopyranosyl-(1→4)-β-D-oleandropyranosyl group by comparing the ^1H and ^{13}C NMR spectra with those of amplexicoside B[11] and confirmed through HMBC correlations of H-1′/C-3, H-1″/C-4′, and H-1‴/C-4″ (Fig. 2). The anomeric configuration of sugars was determined by *J* values at C-1′ (dd, *J* = 10.0,

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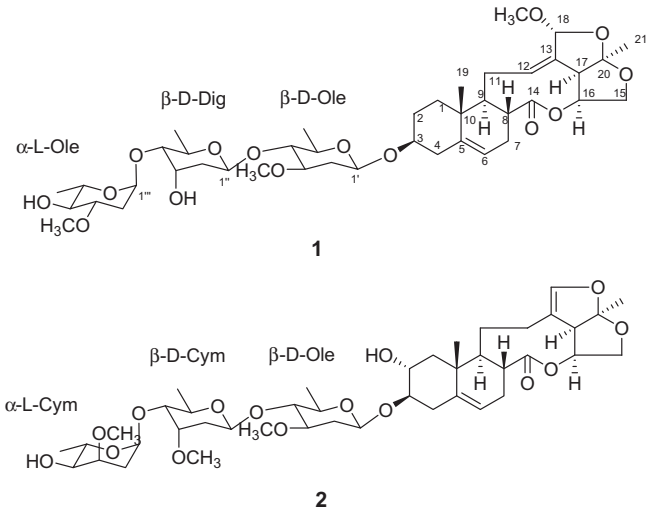

Figure 1. Chemical structures of compounds **1** and **2**.

2.0 Hz), C-1″ (dd, *J* = 10.0, 2.0 Hz), and C-1‴ (d, *J* = 3.0 Hz) as β-, β-, and α-form, respectively. Acid hydrolysis of **1** afforded steroidal sapogenin, cynangenin A (**1a**), which was identified as 3β-hydroxy-18α-methoxy-15,20α:18,20β-diepoxy-13,14:14,15-disecopregna-5,12-dien-14-oic acid 16-oxylactone by ^{1}H NMR, HR-FAB-MS {*m*/*z* 391.2123 $[M+H]^+$ (calcd for $C_{22}H_{31}O_6$, 391.2121)}, and optical rotation {$[\alpha]_D^{25}$ −17.5 (*c* 0.05, MeOH)}, and three sugars (D-oleandrose, L-oleandrose, and D-digitoxose). Identification of D-oleandrose and D-digitoxose was performed by GC/MS analysis,[12] co-TLC with standard samples, and comparison of R_f values with the literature.[13] L-Oleandrose could be identified by comparison of its spectroscopic data with those reported in the literatures.[11,14] The relative configuration of **1** was assumed to be the same as that of stauntogenin 3-*O*-α-oleandropyranosyl-(1→4)-β-digitoxopyranosyl-(1→4)-β-oleandropyranoside based on *J* values [δ_H 5.73 (1H, ddd, *J* = 8.0, 7.5, 5.0 Hz, H-16), 4.42 (1H, dd, *J* = 10.0, 7.5 Hz, H-15a), 4.12 (1H, dd, *J* = 10.0, 5.0 Hz, H-15b)][10] and confirmed by NOESY correlations of H-8/H-19, H-12/H-9 and H-17, H-16/H-17 and H-21, and H-18-OCH_3/H-21 (Fig. 2). Thus, the structure of **1** was determined to be 3β-hydroxy-18α-methoxy-15,20α:18,20β-diepoxy-13,14:14,15-disecopregna-5,12-dien-14-oic acid 16-oxylactone 3-*O*-α-L-oleandropyranosyl-(1→4)-β-D-digitoxopyranosyl-(1→4)-β-D-oleandropyranoside, which was designated cynanside A.

Cynanside B (**2**), an amorphous gum, exhibited a molecular formula of $C_{42}H_{64}O_{15}$ by the molecular ion peak $[M+Na]^+$ at *m*/*z* 831.4144 (calcd for $C_{42}H_{64}NaO_{15}$, 831.4143) by HR-FAB-MS. The ^{1}H and ^{13}C NMR data (Table 2) of the aglycone part of **2** were almost identical to those of cynanoside I[14] and we confirmed the aglycone structure of **2** to be glaucogenin A[14] through DEPT and 2D NMR spectra (^{1}H-^{1}H COSY, HMQC, HMBC, and NOESY). The sugar moiety of **2** was assumed to be α-L-cymaropyranosyl-(1→4)-β-D-cymaropyranosyl-(1→4)-β-D-oleandropyranosyl group observing the similarity of ^{1}H and ^{13}C NMR data to those of stauntoside K[12] except for the presence of a methoxy signal (δ_H 3.55; δ_C 58.3) at C-3″ and upfield shift of C-3″ signal from δ_C 67.9 to 77.8. From the HMBC correlation of H-3″-OCH_3/C-3″ (Fig. 2), we confirmed the location of an additional methoxy group at C-3″. The anomeric configuration of sugars was deduced by *J* values at C-1′ (dd, *J* = 9.5, 2.0 Hz), C-1″ (dd, *J* = 9.5, 2.0 Hz), and C-1‴ (dd, *J* = 4.5, 1.0 Hz) as β-, β-, and α-form, respectively, and the sugar sequences were confirmed through the HMBC correlations of H-1′/C-3, H-1″/C-4′, and H-1‴/C-4″ (Fig. 2). Acid hydrolysis of **2** gave aglycone, glaucogenin A (**2a**), which was identified through ^{1}H NMR and MS spectra with previously reported data,[14] and D-oleandrose, D-cymarose, and L-cymarose. Identification of D-oleandrose was performed as that of **1** and D-cymarose and L-cymarose were to be identified by comparison of their spectroscopic data and R_f

Table 1
^{1}H and ^{13}C NMR spectroscopic data in C_5D_5N for compound **1**[a]

Position	Aglycone		Position	Sugar	
	δ_H	δ_C		δ_H	δ_C
1a	1.71 m	37.1 t	1′(β-D-Ole)	4.81 dd (10.0, 2.0)	98.0 d
1b	1.06 td (13.5, 4.0)		2′a	2.44 m	38.0 t
2a	2.06 br d (13.5)	30.1 t	2′b	1.80 m	
2b	1.66 m		3′	3.60 m	79.1 d
3	3.75 m	77.0 d	4′	3.57 m	83.2 d
4a	2.59 m	38.9 t	5′	3.56 m	71.7 d
4b	2.35 m		6′	1.45 d (6.0)	18.8 q
5		140.0 s	3′-OCH_3	3.57 s	57.5 q
6	5.37 m	120.1 d	1″(β-D-Dig)	5.55 dd (10.0, 2.0)	98.6 d
7a	2.46 m	30.3 t	2″a	2.43 m	39.9 t
7b	2.25 m		2″b	2.00 br t (10.0)	
8	2.47 m	41.3 d	3″	4.61 br s	67.8 d
9	1.98 m	51.9 d	4″	3.54 m	82.3 d
10		37.6 s	5″	4.41 m	68.9 d
11a	2.42 m	29.9 t	6″	1.46 d (6.0)	18.6 q
11b	1.99 m		1‴(α-L-Ole)	5.25 d (3.0)	100.2 d
12	5.48 dd (11.0, 5.0)	131.1 d	2‴a	2.49 m	35.7 t
13		142.2 s	2‴b	1.73 m	
14		179.3 s	3‴	3.84 m	78.7 d
15a	4.42 dd (10.0, 7.5)	70.4 t	4‴	3.53 m	76.8 d
15b	4.12 dd (10.0, 5.0)		5‴	4.43 m	69.4 d
16	5.73 ddd (8.0, 7.5, 5.0)	78.0 d	6‴	1.51 d (6.5)	18.4 q
17	3.29 d (8.0)	56.0 d	3‴-OCH_3	3.33 s	57.0 q
18	5.59 s	104.2 d			
19	0.95 s	19.5 q			
20		114.6 s			
21	1.71 s	24.2 q			
18-OCH_3	3.49 s	55.0 q			

[a] The assignments were based on DEPT, ^{1}H–^{1}H COSY, HMQC, and HMBC experiments. δ in ppm, *J* values in parentheses.

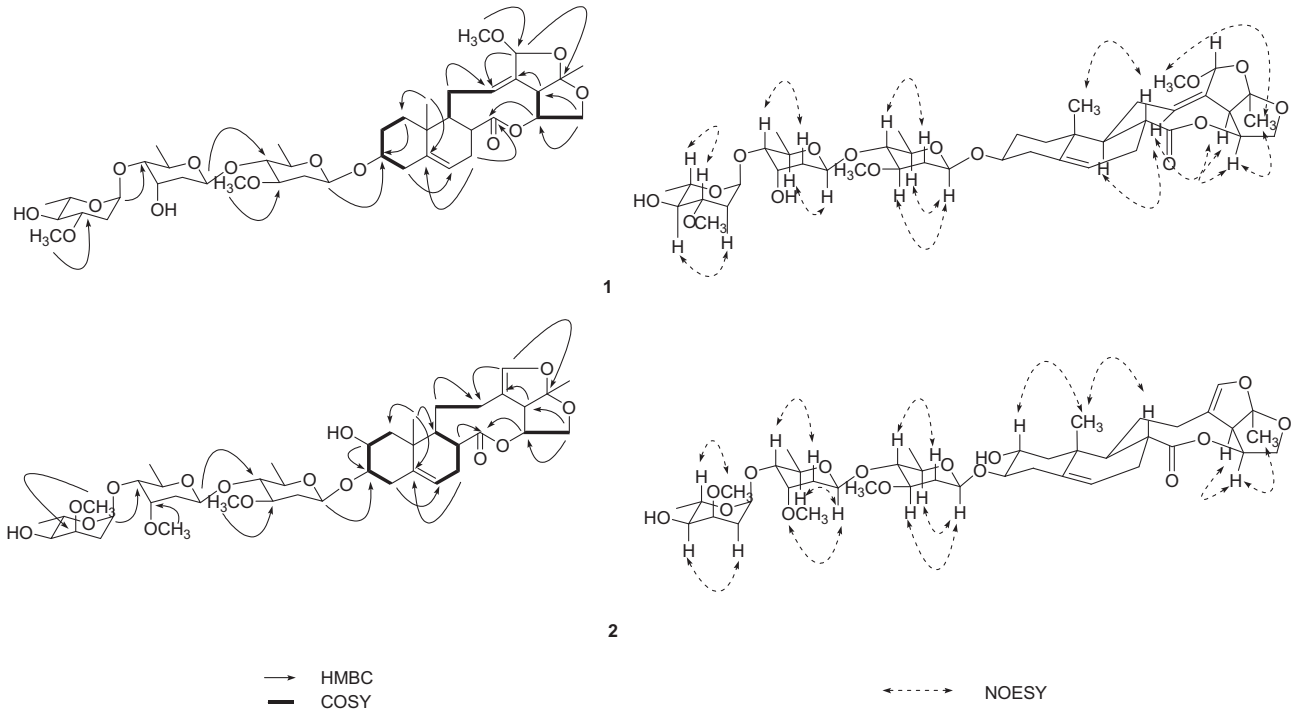

Figure 2. Key HMBC, ^{1}H–^{1}H COSY and NOESY correlations of compounds **1** and **2**.

Table 2
^{1}H and ^{13}C NMR spectroscopic data in C_5D_5N for compound **2**[a]

Position	Aglycone		Position	Sugar	
	δ_H	δ_C		δ_H	δ_C
1a	2.49 m	44.7 t	1′(β-D-Ole)	4.79 dd (9.5, 2.0)	98.9 d
1b	1.27 t (12.0)		2′a	2.45 m	37.6 t
2	4.04 m	69.8 d	2′b	1.79 m	
3	3.66 m	84.9 d	3′	3.54 m	78.9 d
4a	2.57 m	37.3 t	4′	3.49 m	82.4 d
4b	2.49 m		5′	3.57 m	71.8 d
5		139.7 s	6′	1.42 d (6.5)	18.4 q
6	5.47 m	120.8 d	3′-OCH_3	3.54 s	57.8 q
7a	2.66 m	28.4 t	1″(β-D-Cym)	5.26 dd (9.5, 2.0)	98.3 d
7b	2.18 m		2″a	2.29 ddd (13.0, 4.0, 2.0)	36.9 t
8	2.51 m	40.2 d	2″b	1.76 m	
9	1.34 m	53.0 d	3″	3.90 dd (6.0, 2.5)	77.8 d
10		39.5 s	4″	3.45 dd (9.0, 2.5)	82.2 d
11a	2.56 m	23.8 t	5″	4.19 ddd (12.5, 9.0, 6.0)	69.5 d
11b	1.38 m		6″	1.37 d (6.5)	18.5 q
12a	2.13 dd (13.0, 4.0)	30.0 t	3″-OCH_3	3.55 s	58.3 q
12b	1.42 m		1‴(α-L-Cym)	4.98 dd (4.5, 1.0)	98.9 d
13		114.3 s	2‴a	2.38 ddd (15.0, 4.0, 2.0)	32.0 t
14		175.3 s	2‴b	1.82 m	
15a	4.25 dd (9.5, 8.5)	67.7 t	3‴	3.72 dd (7.0, 4.0)	76.3 d
15b	3.95 dd (8.5, 7.0)		4‴	3.61 m	73.3 d
16	5.46 m	75.5 d	5‴	4.55 ddd (13.0, 9.0, 6.5)	66.3 d
17	3.56 m	56.1 d	6‴	1.54 d (6.5)	18.6 q
18	6.49 s	143.8 d	3‴-OCH_3	3.38 s	56.5 q
19	0.93 s	18.9 q			
20		118.5 s			
21	1.55 s	24.7 q			

[a] The assignments were based on DEPT, ^{1}H–^{1}H COSY, HMQC, and HMBC experiments. δ in ppm, J values in parentheses.

values with those reported in the literatures.[11–14] The relative configuration of **2** was determined by NOESY correlations of H-19/H-2 and H-8 and H-16/H-17 and H-21. According to the above analyses, the structure of **2** was elucidated as glaucogenin A 3-*O*-α-L-cymaropyranosyl-(1→4)-β-D-cymaropyranosyl-(1→4)-β-D-oleandropyranose, and was designated cynanside B.

Compounds **3**-**5** were identified by comparing the ^{1}H and ^{13}C NMR, and MS spectra with the literature. They were determined to be paeonol (**3**),[15] 3-hydroxy-4-methoxyacetophenone (**4**),[16] and 3,4-*seco*-lup-20(29)-en-3-oic acid methyl ester (**5**).[17]

All isolates (**1**–**5**) were evaluated for their cytotoxicity against A549 (non small cell lung adenocarcinoma), SK-OV-3 (ovarian

cancer cells), SK-MEL-2 (skin melanoma), and HCT-15 (colon cancer cells) human tumor cell lines using SRB assays in vitro.[18] Compounds **1** and **2** had selective cytotoxicity against SK-MEL-2 cells, with IC_{50} values of 26.55 and 17.36 μM, respectively. However, both compounds were inactive against the other cell lines (IC_{50} >30 μM). The other compounds were inactive against the four tested cell lines (IC_{50} >30 μM).

1. Experimental section

1.1. General methods

Optical rotations were obtained on a JASCO P-1020 polarimeter. IR spectra were recorded on a Bruker Vector 22 IR spectrophotometer. NMR spectra including ^1H–^1H COSY, HMQC, HMBC and NOESY, were recorded on a Varian UNITY INOVA 500 NMR spectrometer operating at 500 MHz (^1H) and 125 MHz (^{13}C) and Bruker AVANCE III 700 NMR spectrometer operating at 700 MHz (^1H). HR-FAB-MS were conducted using a JEOL JMS700 mass spectrometer. Preparative HPLC was performed using a Gilson 306 pump with a Shodex refractive index detector. Silica gel 60 (230–400 mesh, Merck) and RP-C_{18} silica gel (230–400 mesh, Merck) were used for column chromatography. TLC was performed using Merck precoated Silica gel F_{254} plates and RP-18 F_{254s} plates. Spots were detected on TLC under UV light or by heating after spraying with 10% v/v H_2SO_4 in ethanol (EtOH).

1.2. Plant materials

The roots of *C. paniculatum* were collected in Taebaek City, Korea during June 2011. The plant was identified by one of the authors (K. R. Lee). A voucher specimen (SKKU-NPL 1103) of the plant was deposited at the herbarium of the School of Pharmacy at the Sungkyunkwan University, Suwon, Korea.

1.3. Extraction and isolation

The dried roots of *C. paniculatum* (3.6 kg) were pulverized and extracted with 80% MeOH at room temperature. The MeOH extract was concentrated under reduced pressure to afford a viscous concentrate (750 g), which was suspended in water (800 mL) and solvent-partitioned to yield *n*-hexane (75 g), $CHCl_3$ (50 g), EtOAc (9 g), and BuOH (60 g) layers. The $CHCl_3$ layer (20 g) was separated over a silica gel column (230–400 mesh, 500 g) with *n*-hexane:EtOAc:MeOH (3:1:0.5) to give seven fractions (fr. C1–C7). Fr. C3 (4 g) was separated on a RP-C_{18} silica gel column (230–400 mesh, 150 g) with a gradient solvent system of MeOH/H_2O (3:2, 4:1, 9:1, and 1:0) to give seven subfractions (fr. C31–C37). Fr. C33 (700 mg) was further separated over a silica gel column with $CHCl_3$:MeOH (60:1) to give three subfractions (fr. C331–C336). Fr. C332 (100 mg) was purified with a silica gel prep. HPLC with $CHCl_3$:MeOH (60:1) at a flow rate of 2.0 mL/min to give **1** (4 mg, t_R = 19.1 min). Fr. C34 was separated over a silica gel column with $CHCl_3$:MeOH (65:1) and further purified by a silica gel prep. HPLC with $CHCl_3$:MeOH (80:1) to yield **2** (7 mg, t_R = 15.4 min). The *n*-hexane layer (20 g) was separated over a silica gel column (230–400 mesh, 500 g) with *n*-hexane:EtOAc (15:1) to give eight fractions (fr. H1–H8). Fr. H1 (890 mg) was further separated over a Sephadex LH-20 column with CH_2Cl_2:MeOH (1:1) and purified by a silica gel prep. HPLC with *n*-hexane:EtOAc (12:1) to yield **5** (22 mg, t_R = 13.4 min). Fr. H2 was separated over a RP-C_{18} silica gel column with 75% MeOH, Sephadex LH-20 column with CH_2Cl_2:MeOH (1:1), and RP-C_{18} silica gel prep. HPLC with 50% CH_3CN to yield **3** (19 mg, t_R = 16.3 min). Fr. H8 was further separated over a RP-C_{18} silica gel column with 75% MeOH and purified by a silica gel preparative HPLC with *n*-hexane:EtOAc (2:1) to yield **4** (7 mg, t_R = 17.2 min).

1.3.1. Cynanside A (1)

Amorphous gum, $[\alpha]_D^{25}$ −6.0 (*c* 0.05, MeOH); IR (KBr) ν_{max}: 3416, 3080, 3029, 1647, 1582, 1213, 1148, 1069, 1028 cm^{-1}; ^1H NMR (C_5D_5N, 500 MHz) and ^{13}C NMR (C_5D_5N, 125 MHz) see Table 1; HR-FAB-MS *m*/*z* 831.4144 $[M+Na]^+$ (calcd for $C_{42}H_{64}NaO_{15}$, 831.4143).

1.3.2. Cynanside B (2)

Amorphous gum, $[\alpha]_D^{25}$ −34.0 (*c* 0.05, MeOH); IR (KBr) ν_{max}: 3420, 3082, 3030, 1650, 1580, 1215, 1146, 1069, 1028 cm^{-1}; ^1H NMR (C_5D_5N, 500 MHz) and ^{13}C NMR (C_5D_5N, 125 MHz) see Table 2; HR-FAB-MS *m*/*z* 831.4144 $[M+Na]^+$ (calcd for $C_{42}H_{64}NaO_{15}$, 831.4143).

1.4. Acid hydrolysis of 1 and 2

Compound **1** (1.0 mg) was heated at 60 °C and stirred with 1 mL of 0.02 N H_2SO_4 for 1 h. After cooling, the hydrolysate was extracted with $CHCl_3$ and the extract was evaporated in vacuo to yield a compound **1a** as an amorphous gum. Compound **2** was treated similar as **1** to yield **2a.**

1.4.1. Cynangenin A (1a)

Amorphous gum, $[\alpha]_D^{25}$ −17.5 (*c* 0.05, MeOH); ^1H NMR ($CDCl_3$, 700 MHz): δ_H 5.72 (1H, m, H-16), 5.50 (1H, s, H-18), 5.49 (1H, m, H-12), 5.32 (1H, m, H-6), 4.29 (1H, dd, *J* = 10.3, 7.4 Hz, H-15a), 3.88 (1H, dd, *J* = 10.3, 5.3 Hz, H-15b), 3.59 (1H, m, H-3), 3.45 (3H, s, H-18-OCH_3), 1.59 (3H, s, H-21), 0.99 (3H, s, H-19); HR-FAB-MS *m*/*z* 391.2123 $[M+H]^+$ (calcd for $C_{22}H_{31}O_6$, 391.2121).

1.4.2. Glaucogenin A (2a)

Amorphous gum, $[\alpha]_D^{25}$ +74.5 (*c* 0.35, MeOH); ^1H NMR (C_5D_5N, 500 MHz): 6.48 (1H, s, H-18), 5.44 (1H, m, H-16), 5.41 (1H, m, H-6), 4.25 (1H, dd, *J* = 10.0, 8.5 Hz, H-15a), 3.96 (1H, dd, *J* = 8.5, 7.0 Hz, H-15b), 3.56 (1H, m, H-17), 1.54 (3H, s, H-21), 0.99 (3H, s, H-19); FAB-MS *m*/*z* 399.2 $[M+Na]^+$.

1.5. Determination of the absolute configurations of sugars

The sugar obtained from the hydrolysis of **1** was dissolved in anhydrous pyridine (0.5 mL) followed by adding of L-cysteine methyl ester hydrochloride (2.0 mg), and the mixture was stirred at 60 °C for 1.5 h. Acetic anhydride (0.5 mL) was added and heated for another 1 h. The mixture was partitioned between *n*-hexane and H_2O (1.0 mL each), and the organic layer (1.0 μL) was analyzed by GC/MS. Identification of D-oleandrose and D-digitoxose was performed by co-injection of the hydrolysate with standard acetylated samples (D-oleandrose, hydrolyzed from cynapanoside A;[10] D-digitoxose, Sigma–Aldrich), giving a single peak at 6.238 min (D-oleandrose) and 9.476 min (D-digitoxose). Compound **2** (1.0 mg) was treated as above and yielded aglycone **2a** and sugars. D-Oleandrose was identified by GC/MS (R_t = 6.268 min). GC conditions in the test: column, HP-5MS UI, 30 m × 0.25 mm, 0.25 μm; detection, MS; carrier gas, He; injection temperature, 250 °C, detection temperature, 280 °C, column temperature, 150 °C (0 min), 10 °C/min to 250 °C (20 min). R_t D-oleandrose 6.246 min.

D-Oleandrose, L-oleandrose, D-cymarose, L-cymarose, and D-digitoxose were identified by co-TLC with standard samples and comparison of R_f values with the literature;[13] D,L-oleandrose ($CHCl_3$:MeOH = 8:1, R_f value: 0.48), D, L-cymarose ($CHCl_3$:MeOH = 8:1, R_f value: 0.52), and D-digitoxose ($CHCl_3$:MeOH = 8:1, R_f value: 0.40).

1.6. Cytotoxicity assay

A SRB bioassay was used to determine the cytotoxicity of each compound isolated against four cultured human tumor cell lines.[18] The assays were performed at the Korean Research Institute of Chemical Technology. The cell lines used were A549, SK-OV-3, SK-MEL-2, and HCT-15. Doxorubicin was used as a positive control. The cytotoxicities of doxorubicin against the A549, SKOV-3, SK-MEL-2, and HCT-15 cell lines were IC_{50} 0.029, 0.036, 0.001, and 2.041 μM, respectively.

Acknowledgements

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Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.carres.2013.08.023.

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